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Biochimica et Biophysica Acta 1497 (2000) 175–185

BIOCHIMICA ET BIOPHYSICA ACTA

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Involvement of $p27^{kip1}$ and cyclin D3 in the regulation of cdk2 activity during skeletal muscle differentiation

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Received 3 February 2000; received in revised form 23 May 2000; accepted 26 May 2000

Abstract

Terminal myogenic differentiation involves an irreversible transition from a proliferative state to a post-mitotic quiescent state. We showed here that in addition to the previously reported down regulation of G_1-related cyclin-associated kinase activities, this transition was also accompanied by an extensive reorganization of the cyclin–cdk complexes, including a dramatic shift of cdk2 from cyclin A to cyclin D3. Moreover, the inhibition of cdk activity also correlated with an increase in the expression of the $p27^{kip1}$ cdk inhibitor and in its association with the cyclin–cdk2 complexes. Since depletion of p27 substantially reduced the cdk inhibitor activity present in differentiated muscle cells, we believe that the increase in p27 expression along with the reorganization of the cyclin–cdk2 complexes may play an important role in the inhibition of cdk2 activity during the differentiation process.

Keywords: Cell cycle; Cyclin-dependent kinase; Terminal differentiation; Growth regulation; Cdk inhibitor

1. Introduction

Terminal differentiation of skeletal muscle cells is normally accompanied by a permanent and irreversible withdrawal of the cells from active proliferation into a postmitotic state that is unresponsive to subsequent mitogenic stimulation [1,2]. Differentiated cells contain a diploid content of nuclear DNA and appear to reside in a G_0 state. This postmitotic state has been correlated with the hypophosphorylation of the retinoblastoma protein RB and a drastic decrease in both cyclin-dependent kinase 2 (cdk2) and cyclin-dependent kinase 4 (cdk4) activity [2–5]. The precise mechanism for the loss of cdk activity has not been completely defined. A number of studies have implicated the $p21^{cip1}$ and the $p18^{ink}$ cdk inhibitors, respectively, in the suppression of cdk2 and cdk4 activities [3,4,6,7]. However, transgenic knockout mice deficient in p21 expression did not manifest any developmental abnormality in skeletal muscle formation [8] suggesting that normal p21 function is dispensable or at least redundant for normal myogenesis [8]. On the other hand, although an increase in the $p27^{kip1}$ inhibitor expression during in vitro myogenesis has been observed [3,6], its involvement in the generation and maintenance of the growth-arrested state has not been addressed. Similarly, while changes in the expression level of various G_1-related cyclins have been analyzed [5,9,10], how they might impinge on the postmitotic regulation of

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0167-4889/00/$ – see front matter
PII: S0167-4889(00)00064-1

differentiated muscle cells is not completely understood. Using the skeletal muscle cell line C2C12 as a model system, we have analyzed cdk2 activity and the kinase activity associated with three G_1-related cyclins: cyclin A, cyclin E and cyclin D3 [11–16]. We have also monitored the expression level of cdk2 and these cyclins and their intermolecular associations with each other and with the cdk inhibitor $p27^{kip1}$ [17]. Our data suggest that in addition to the well-documented increase in $p21^{cip1}$, the postmitotic terminally differentiated state might also involve a redistribution of cdk2 from cyclin A to cyclin D3 as well as an increased interaction of cyclin D3 and cdk2 with the p27 cdk inhibitor. The cumulative evidence thus argued that the entry and maintenance of the postmitotic state requires both the redistribution of cyclin–cdk complexes and the involvement of multiple cdk inhibitors.

2. Materials and methods

2.1. Cell culture

C2C12 and 10T1/2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 40 μg/ml gentamycin in a 37°C humidified incubator under a 5% CO_2 atmosphere. The myogenic subclone of 10T1/2 cells (Cl3f) was derived from the parental 10T1/2 cells by stable transfection of the MyoD containing expression plasmid pEMC11 [18] and isolated by G418 selection. Differentiation of the myogenic cells was initiated by exposing cells at about 50% confluence to DMEM containing 5% horse serum. Under such conditions, morphological and biochemical differentiation generally became detectable after 2 days in the differentiation-permissive medium with more than 50% of the cells undergoing differentiation by day 4 [19,20]. Unless otherwise stated, the differentiated cell extracts used in the studies were harvested on day 4 after the medium switch.

2.2. Immunoprecipitation and immunoblot

The antibodies used for the study include: anti-cyclin A antibody H-432, anti-cyclin D1 antibody 73-13G, anti-cyclin D3 antibody C-16, anti-cyclin E antibody M-20, anti-cdk2 antibody M-2, and anti-p27 antibody C-19 (purchased from Santa Cruz). For immunoprecipitation, cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 1 mM EDTA, containing 0.5% NP40, 2 μg/ml aprotinin, 0.2 mM PMSF, 2 μM leupeptin and 1 μM pepstatin) for 15 min on ice. Nuclei were removed by centrifuging the cell lysate at 11 000 rpm for 5 min. The lysate was incubated with various primary antibodies for 1 h on ice. Ten μl of 50% protein A agarose (Boehringer Mannheim) was then added to each sample tube and the incubation was continued at 4°C for 1 h with rocking, followed by centrifugation to isolate the immunocomplexes. For immunoblot analysis, cell lysates or immunoprecipitates were separated by SDS–PAGE and transferred to nitrocellulose membrane using a Bio-Rad mini-blot electrotransfer apparatus in transfer buffer containing 25 mM Tris, 192 mM glycine, 0.075% SDS and 20% methanol. The membrane was incubated in Western wash buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween) containing 2% milk to block non-specific binding, followed by incubation with primary antibodies diluted in Western wash buffer containing 2% milk at the appropriate dilution for each antibody. The membrane was wash extensively with Western wash buffer and then incubated with horseradish peroxidase-conjugated antibody (Bio-Rad, 1:2000–4000 dilution) and detected by enhanced chemiluminescence system (Amersham, Dupont and Pierce).

2.3. Cdk2 kinase activity assay

Cdk2 kinase assay was performed on immunocomplexes from cell lysates as described by Guo et al. with slight modifications [4]. Briefly, the protein A agarose beads containing the immunocomplexes were washed three times with 500 μl of lysis buffer and twice with 500 μl kinase buffer (50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT). Fifty microliters of kinase buffer containing 0.05 mg/ml histone, 10 mM ATP and 10 μCi γ-^{32}P-ATP was added to the agarose beads. After incubation at room temperature for 20 min, the reaction was terminated by adding 50 μl of 2× sample buffer. Samples were separated by SDS–gel and the dried gel was exposed to X-ray film. For experiments involving the inhibition of cdk2 activity by heat-released factor(s), total cell lysates or

immunocomplexes resuspended in lysis buffer were boiled for 4 min and then centrifuged at 10 000 rpm for 5 min at 4°C. The supernatants from the centrifugation step were added to proliferating cell lysates and incubated for 30 min on ice. Cdk2 activity was then assayed following immunoprecipitation by anti-cdk2 antibody.

2.4. Immunodepletion of p27

Immunoprecipitation was performed as described above using either 5 μl of anti-p27 antibody (0.1 mg/ml) or normal rabbit IgG. The supernatant separated from the protein A agarose beads by centrifugation were immunoprecipitated with the same amount of antibody two more times. Each immunoprecipitate was analyzed by SDS–PAGE and immunoblot with anti-p27 antibody to check for the degree of p27 depletion. The final supernatant was used for inhibition of cdk2 activity as described above.

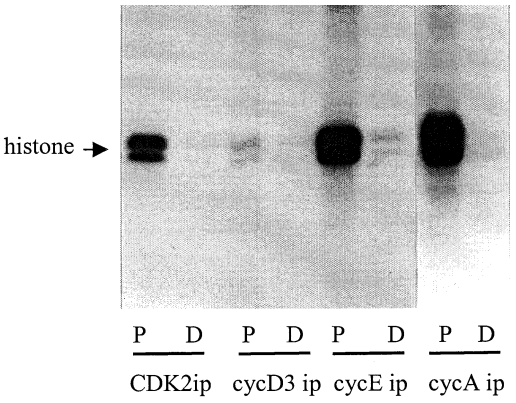

Fig. 1. Differentiated C2C12 cells contained decreased levels of cdk2 activity and histone kinase activity associated with various G1 cyclins. Cell extracts from proliferating (P) and differentiated (D) C2C12 cells were subjected to immunocomplex kinase assays using histone as substrate following immunoprecipitation with anti-cdk2 antibody (cdk2 ip), anti-cyclin D3 antibody (cyc D3 ip), anti-cyclin E antibody (cyc E ip) or anti-cyclin A antibody (cyc A ip) as described in Section 2. The samples were then analyzed by SDS–PAGE and autoradiography. The arrow indicates where the phosphorylated histone migrates on the gel.

3. Results

3.1. A low level of cyclin D3 associated kinase activity is present in proliferating myoblasts, but not in differentiated muscle cells

Previous studies have shown that terminally differentiated muscle cells expressed little or no cdk2 or cdk4 activity [2–5]. Using antibodies directed against cdk2 and a number of G_1-related cyclins to precipitate kinase activity from C2C12 skeletal muscle cells, we have confirmed that differentiation was correlated with a decrease in immunoprecipitable cdk2 activity and kinase activities associated with cyclin E and cyclin A (Fig. 1). Interestingly, we also detected a low cyclin D3-associated kinase activity in proliferating cells, which showed a further decline following muscle cell differentiation. This D3-associated kinase activity is most likely contributed by D3–cdk2 complexes, since previous studies have shown that cdk4 does not phosphorylate histone efficiently in vitro [21,22]. Consistent with this idea, we could not detect histone kinase activity in our anti-cdk4 immunoprecipitates and no cyclin D1-associated histone kinase was detected in either proliferating or differentiated cells (data not shown).

3.2. The decrease in cyclin D3 associated kinase activity was accompanied by a paradoxical but muscle-differentiation-specific increase in cyclin D3 expression

Previous studies have shown that muscle differentiation in several in vitro systems was accompanied by changes in the expression levels of various cyclins, including a paradoxical increase of cyclin D3 [5,9]. Results from our study confirmed small decreases in the levels of cyclin E, cyclin A and cdk2, a relatively large decrease in the level of cyclin D1 and an equally striking increase in cyclin D3 in differentiated C2C12 cells (Fig. 2A).

In addition, we have extended the observation to demonstrate that the increase in cyclin D3 was specific to the myogenic differentiation process. A myogenic subclone (Cl3F) of the fibroblastic 10T1/2 embryonic cell line was generated by stable transfection of a MyoD1 expressing vector and the cyclin D3 levels of Cl3f cells and the parental 10T1/2 cells were examined following exposure to differentiation-inducing conditions. Whereas the parental 10T1/2 cells which could not differentiate did not exhibit any increase in cyclin D3 level, the Cl3f cells exhibited a pronounced increase in cyclin D3 when

'induced' to differentiate (Fig. 2B). Mock transfected 10T1/2 clones or 10T1/2 clones transfected with unrelated plasmids also did not exhibit any increase in cyclin D3 levels (data not shown). The increase in cyclin D3 protein thus appears to be an intrinsic part of the differentiation program that can be activated by the ectopic overexpression of a single myogenic regulatory gene.

3.3. The increase in cyclin D3 expression was accompanied by its increased association with cdk2

We examined next whether the association between the various cyclins and cyclin-dependent kinases might be altered during the differentiation process. We showed that antibody to cyclin E precipitated similar amounts of cdk2 from both proliferating and differentiated cells (Fig. 3A) while the amount of cyclin D3 co-precipitating with cdk2 increased following cell differentiation (Fig. 3B). This increase in interaction between cyclin D3 and cdk2 was again specific to the muscle differentiation process since increased association was only observed with the myogenic subclone Cl3f following differentiation and not with parental 10T1/2 cells subjected to similar culture conditions (Fig. 3C, lanes 3–7). In contrast, cdk2 was detected in co-immunoprecipitates with antibody to cyclin A from proliferating cells, but not from differentiated cells (Fig. 3D) and little to no cyclin D1 was detected in anti-cdk2 immunoprecipitates from either the proliferating or differentiated muscle cells (Fig. 3E).

3.4. Expression of the heat-stable cdk inhibitor $p27^{kip1}$ increased in the differentiated cells and became associated with specific cyclin and cdk complexes

The p21 family of cdk inhibitors: $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ had been shown to bind and inhibit both cdk2 and cdk4 activities in vitro [23,24]. Although p27 is highly expressed in differentiated muscle cells [6], its potential involvement in muscle differentiation has largely been overlooked, in part because p27 expression also increased as cells became quiescent [17,25]. We found that the level of p27 was indeed

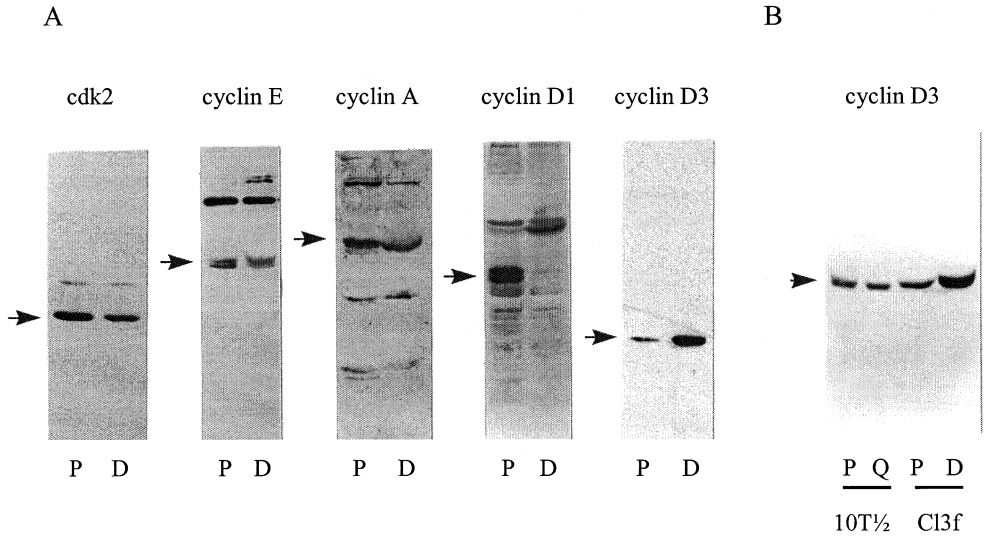

Fig. 2. Expression levels of cdk2 and various G1 cyclins in C2C12 and other myogenic and non-myogenic cell lines. Total cell extracts normalized to contain equal amounts of proteins were electrophoresed on SDS–PAGE, transferred to nitrocellulose and probed with specific antibodies against cdk2 and the indicated cyclins, followed by immunodetection with enhanced chemiluminescence as described in Section 2. The arrows mark the expected positions of either cdk2 or the particular cyclin indicated on top of each gel as determined by comparison with molecular size markers. Panel A demonstrates that muscle differentiation was accompanied by differential changes in the level of expression of cdk2 and various G1 cyclins. Lane P, extracts from proliferating cells. Lane D, extracts from differentiated cells. In panel B, cyclin D3 expression was examined in cultures of the non-myogenic 10T1/2 cells or a myogenic subclone of these cells (Cl3f). When subjected to the differentiation-inducing conditions, the Cl3f cells undergo myogenic differentiation (D) while the 10T1/2 cells became quiescent (Q) but were unable to differentiate.

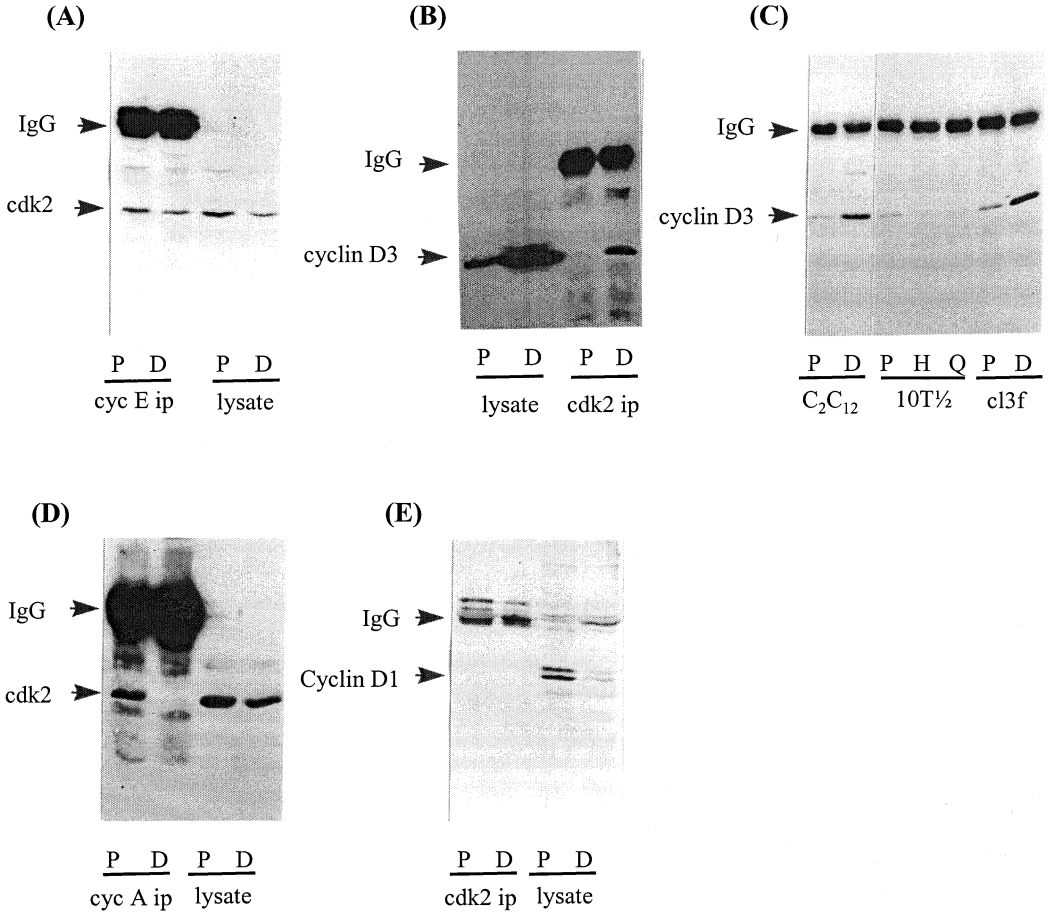

Fig. 3. Co-immunoprecipitation of various cyclins with cdk2 in C2C12 and other myogenic and non-myogenic cell lines. Total cell lysates were subjected to immunoprecipitation with either anti-cdk2 or anti- cyclin antibody. The immunoprecipitates were then analyzed by immunoblotting with the complementary antibody to look for co-precipitation between cdk2 and the specific cyclin. For comparison, an aliquot of the total lysate (15% of the amount used in the immunoprecipitation) was electrophoresed directly and analyzed by immunoblot (Lanes 3 and 4 in panels A, D, and E; lanes 1 and 2 in panel B). Except for panel C, the amount of lysates from proliferating (P) and differentiated (D) cultures were adjusted to contain the same amount of the antigenic protein being precipitated (cyclin E for panel A, cdk2 for panels B and E, and cyclin A for panel D). (A) Anti-cyclin E antibody precipitated cdk2 from both proliferating and differentiated C2C12 cells. (B) Anti-cdk2 antibody precipitated more cyclin D3 from differentiated C2C12 cells. (C) Anti-cdk2 antibody precipitated increased amounts of cyclin D3 only in differentiation-competent cells. Lysates from C2C12, and the non-myogenic 10T1/2 and myogenic Cl3f were normalized to contain the same amount of cyclin D3 prior to immunoprecipitation with the anti-cdk2 antibody. Lane H represents immunoprecipitates from 10T1/2 cells that were exposed to culture condition used to induce differentiation in the other cell types. Lane Q represents 10T1/2 cells that had become quiescent and were no longer proliferating. (D) cdk2 was precipitated by anti-cyclin A antibody from proliferating C2C12 cell lysates, but not from differentiated C2C12 cell lysates. (E) Anti-cdk2 antibody did not precipitate cyclin D1 either in proliferating or differentiated C2C12 cells.

low in subconfluent proliferating C2C12 cells, and increased rapidly as the cultures were exposed to 'differentiation-inducing' conditions (Fig. 4A). The expression was maintained at a raised level following differentiation, which typically began after 2 days in the differentiation medium.

We examined next the association of p27 with various cyclins and cdks. Lysates from proliferating and differentiated C2C12 cells were immunoprecipitated with antibodies directed against specific cyclins and cdks and the presence of p27 in the immunoprecipitate was analyzed by immunoblotting with anti-p27 antibody. While some p27–cyclin D3 complexes were detectable in the proliferating cell lysates, the amount of p27 protein associated with cyclin D3 increased substantially following differentiation (Fig. 5A). Sim-

ilarly, binding between cdk2 and p27 was virtually undetectable in the proliferating cells, but became clearly evident following differentiation. Likewise, p27 protein could be co-precipitated with the anti-cyclin E antibody only following cellular differentiation. In contrast, no p27 could be detected in the cyclin A immunoprecipitates from either the proliferating or differentiated cell lysates (Fig. 5B).

3.5. *Differentiated muscle cell lysates contained a cdk inhibitory activity that could be depleted by immunoprecipitation with anti-p27 antibodies*

Previous studies have shown that the cdk-inhibitory activity of $p27^{kip1}$ is heat stable and can be freed from associated proteins upon heat denaturation [4,17,25]. We showed that differentiated C2C12 cell lysates heated to 100°C indeed released $p27^{kip1}$ (Fig. 4B) and a cdk-inhibitor activity that could block the kinase activity of cdk2 from proliferating cells (Fig. 4C).

To assess the nature of the cdk2 inhibitory activity further, we incubated differentiated cell lysates separately with either anti-cyclin D3 or anti-p27 antibodies and protein A agarose beads, and heat-denatured the immunoprecipitates to see if inhibitory activity would be released from the immune complexes. Our results demonstrated a substantial amount of cdk2 inhibitory activity could be recovered from these complexes (Fig. 4D, lanes 2 and 3), whereas no inhibitory activity was recovered when non-immune IgG was used (Fig. 4D, lane 4). It thus ap-

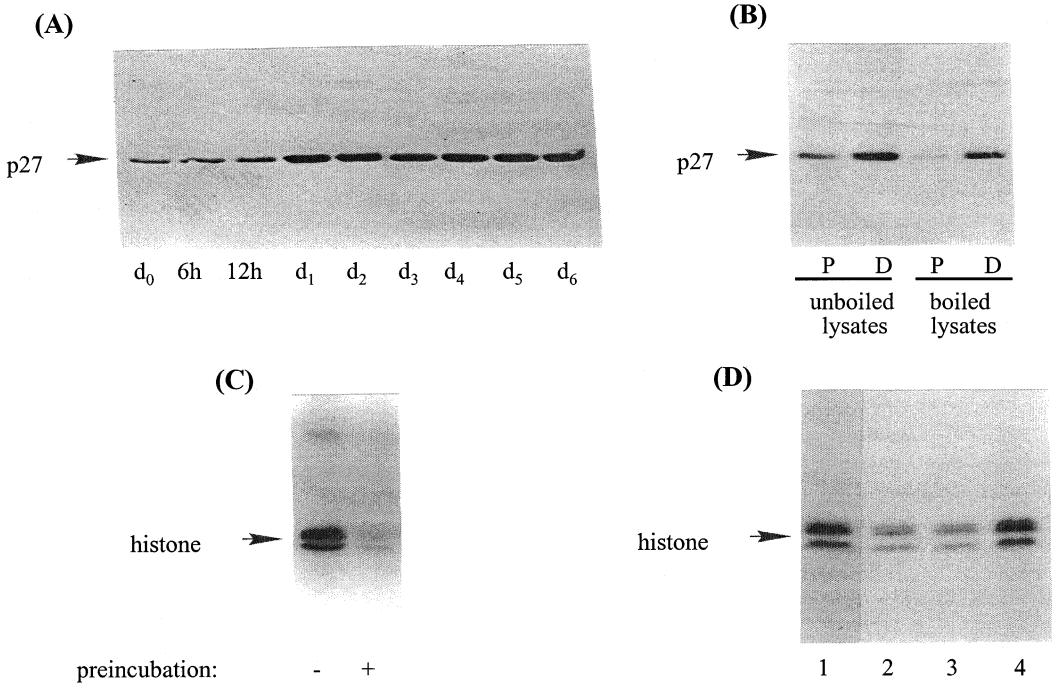

Fig. 4. Expression of p27 and cdk2 inhibitory activity in C2C12 cells. (A) Expression of p27 protein increased with increasing time in differentiation-inducing conditions. Total lysates from C2C12 cells exposed to differentiation-inducing conditions for the indicated lengths of time were analyzed by immunoblotting with anti-p27 antibody. (B) Release of p27 protein from both proliferating and differentiated C2C12 cells into the supernatant by boiling. Total lysates (lanes 1 and 2) or supernatant from lysates that have been boiled (lanes 3 and 4) were analyzed by immunoblotting with the anti-p27 antibody. Lane P, lysates from proliferating cells. Lane D, lysates from differentiated cells. (C) Boiling released a cdk2 inhibitory activity from differentiated cell extract. Extracts from proliferating C2C12 cells were analyzed for cdk2 activity by the immunocomplex kinase assay described in Section 2 (lane 1), or preincubated with boiled and clarified lysate from differentiated cells before immunoprecipitation with the anti-cdk2 antibody (lane 2). (D) the inhibitory activity can be immunoprecipitated by anti-p27 and anti-cyclin D3 antibody. Total lysates from differentiated C2C12 cells were incubated with anti-p27 (lane 2), anti-cyclin D3 antibody (lane 3) or non-immune IgG (lane 4) followed by protein A agarose. Materials released from the agarose beads after boiling were assayed for cdk2 inhibitory activity as described for panel C. Lane 1 represents the cdk2 activity precipitated by anti-cdk2 antibody from proliferating cell lysate with no cdk inhibitors added.

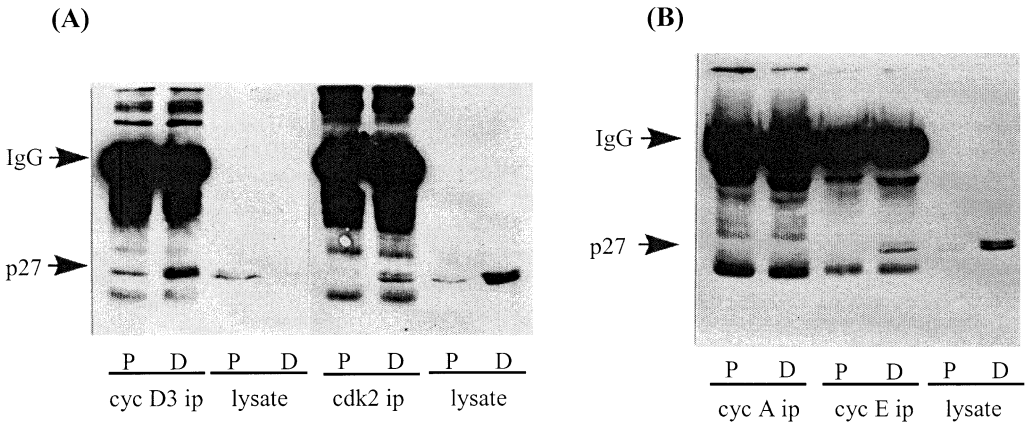

Fig. 5. Association of p27 with cdk2 and various cyclins. Total cell lysates from proliferating (P) and differentiated (D) cultures were subjected to immunoprecipitation with the specific anti-cdk2 or anti-cyclin antibody indicated. The immunoprecipitates were then analyzed by immunoblotting with anti-p27 antibody. For comparison, an aliquot of total lysates (15% of the amount used in the immunoprecipitation) was electrophoresed directly and analyzed by immunoblot (lysate lanes). Each pair of lysates was adjusted so as to contain approximately the same amount of cyclin or cdk2 targeted by the immunoprecipitating antibody. (A) p27 associated with cyclin D3 in lysates from both proliferating and differentiated cultures, but only with cdk2 in the differentiated cultures. Note that because of the large increase in cyclin D3 expression during differentiation, a much larger amount of proliferating cell extract was used in the immunoprecipitation, leading to the apparent reduction of p27 level in the differentiated cell lysate. (B) p27 associated with cyclin E in differentiated culture lysates, but not with cyclin A, in either proliferating or differentiated cultures.

peared that at least part of the inhibitory activity in the differentiated cells corresponds to the p27 protein and is associated with cyclin D3 in differentiated muscle cells.

To determine what extent of the inhibitory activity observed was attributable to p27, we depleted p27 by repetitive immunoprecipitation with anti-p27 antibody and examined the lysates for residual cdk-inhibitory activity. As shown in Fig. 6A, the anti-p27 precipitates contained very little p27 protein by the third round of immunoprecipitation indicating that the p27 protein had been depleted from the lysates. Coincident with this loss of p27 immunoreactivity, the level of cdk2 inhibitory activity that could be released from the cell lysate upon heating was also dramatically reduced (Fig. 6B, lane 4). The reduction of cdk2 inhibitory activity was not due to non-specific absorption of the cdk2 inhibitor(s) to the agarose beads used in the immunoprecipitation, since lysates subjected to the same precipitation protocol using non-immune IgG retained full inhibitory activity (Fig. 6B, lane 3).

4. Discussion

Using the murine myogenic C2C12 cells as our model system, we have investigated the molecular mechanisms involved in regulating cell cycle withdrawal in differentiating skeletal muscle cells. Our results confirmed that cdk2 kinase activity was drastically diminished upon muscle differentiation [3,4] and that the differentiation process was accompanied by changes in the expression levels of multiple components of the cell cycle regulatory machinery, including the down regulation of cdk2, cyclin A, cyclin D1 and cyclin E, and the upregulation of cyclin D3 and $p27^{kip1}$. While the exact magnitudes of the changes in some of the individual components may differ slightly with those reported in some studies, the general pattern we observed is consistent with previous findings (reviewed in Walsh and Perlman [26]).

In addition to confirming these findings, our study has extended the previous observations by demonstrating that a drastic reorganization of the cyclin–cdk complexes also takes place during skeletal muscle differentiation (summarized schematically in Fig. 7). The most notable aspect of this reorganization is the striking decrease in cdk2 binding to cyclin A and a corresponding increase in binding to cyclin D3. In contrast, the cyclin E–cdk2 complex remained intact, but appeared to have become increasingly associated with the cdk inhibitor $p27^{kip1}$. We corroborated these physical findings by showing that both

cyclin A- and cyclin E-associated kinase activities were greatly reduced. Our data thus demonstrated that although myogenic differentiation was accompanied by the coordinate extinguishing of cdk activities from two cyclin–cdk complexes that are both important for entry into S phase [13,14,27], the decrease in kinase activity is accomplished by different molecular mechanisms: decreased binding to cdk2 in the case of cyclin A and increased binding of cdk inhibitors in the case of cyclin E.

While the paradoxical increase in cyclin D3 expression during muscle differentiation has been reported previously [5,9,28], its increased association with cdk2 and with the cdk inhibitor $p27^{kip1}$ has never been documented. In this regard, although interactions between cyclin D3 and cdk2 could occur in vitro and D3–cdk2 complexes have been detected in some cells [29,30], it is widely believed that cyclin D3 exerts its regulatory effects primarily through cdk4. However, since results from the current study clearly demonstrated that both the increase in cyclin D3 expression and its increased interaction with cdk2 were highly specific to the muscle differentiation process, the possibility that the D3–cdk2 interaction may have important biological relevance can no longer be ignored (see further discussion below).

Another novel finding in this study is the involvement of p27 in regulating cdk2 activity in differentiated skeletal muscle cells. Previous studies have implicated increased expressions of other cdk inhibitors, including $p18^{ink4c}$ [3] and $p21^{cip1}$ [4,6,7] respectively, in the inhibition of cdk4 and cdk2 activities during skeletal muscle differentiation. In addition, the involvement $p57^{kip2}$ is suggested by p57 genetic 'knockout' mice [31,32] and by the recent report that mice with combined deficiencies in p57 and p21 exhibit a dramatic defect in skeletal muscle development [33]. On the other hand, our immunodepletion experiment clearly indicated that a significant portion of the heat-stable cdk2 inhibitory activity present in differentiated muscle cells was contributed by $p27^{kip1}$ since the anti-p27 antibody

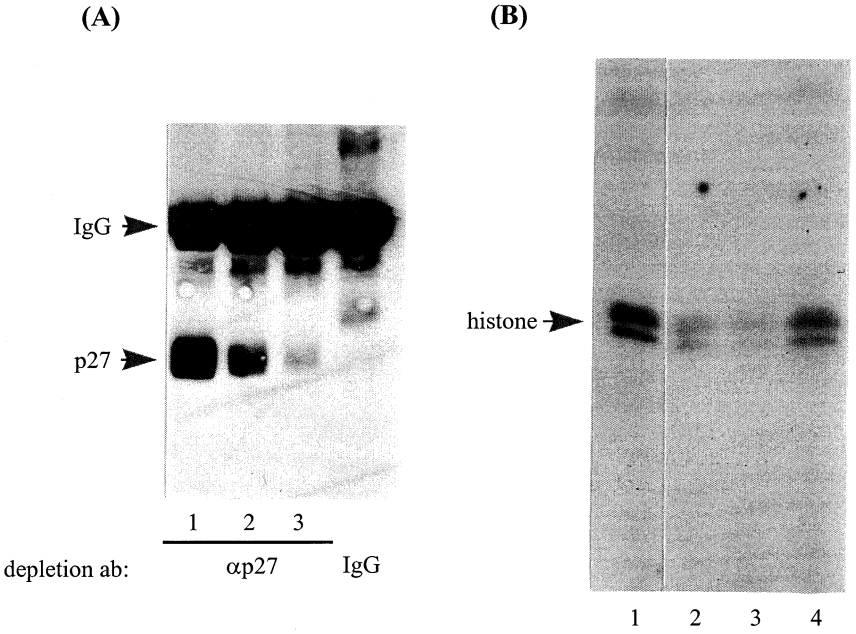

Fig. 6. Immunodepletion of p27 removes the cdk2 inhibitory activity from differentiated C2C12 lysates. Total lysates form differentiated C2C12 cultures were subjected to three rounds of immunoprecipitation with anti-p27 antibody or with non-immune IgG. In panel A, the immunoprecipitates were analyzed by immunoblot with anti-p27 antibody. Lanes 1, 2 and 3 correspond to anti-p27 immunoprecipitates from the first, second and third round of precipitation. The precipitates from the first round of precipitation using the non-immune IgG are shown in lane 4. In panel B, the supernatant after the third round of precipitation was boiled and the released material was assayed for inhibitory activity as described in Fig. 4. Lane 1 corresponds to control cdk2 activity precipitated directly from untreated proliferating cell lysate; lane 2 corresponds to cdk2 activity from a sample that had been preincubated with undepleted boiled lysate from differentiated cells; lane 3 represents cdk2 activity from sample preincubated with non-immune IgG 'depleted' lysate; lane 4 represents cdk2 activity from sample preincubated with anti-p27 depleted lysate.

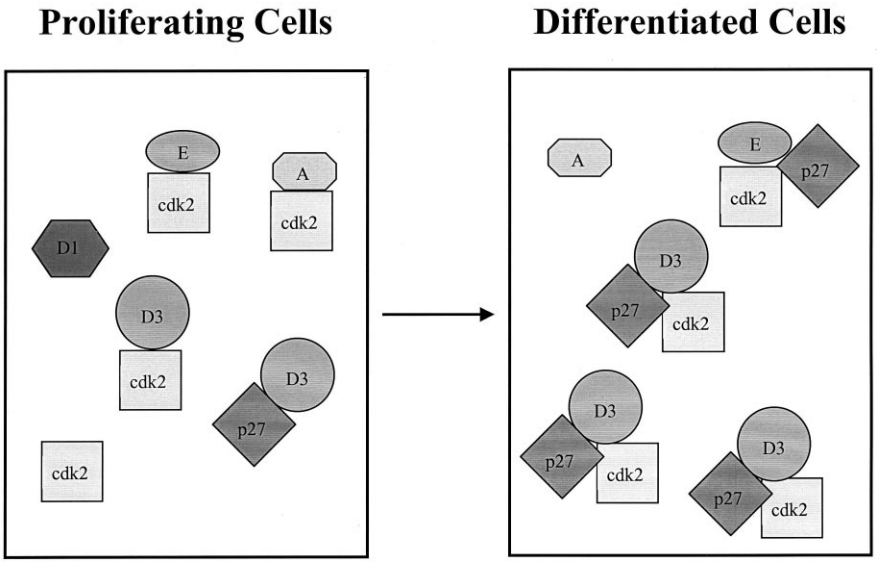

Fig. 7. Model depicting how the decline of cdk2 activity might be regulated by increased p27 and cyclin D3 expression and the reorganization of the cdk2'cyclin complexes. Findings from these studies documented an increase in cyclin D3 and p27 expression and interaction during muscle differentiation. This is postulated to result in the sequestration of cdk2 from cyclin A into inactive D3–p27–cdk2 complexes. The cyclin E-associated cdk2 activity was also blocked by interaction with p27, resulting in the virtually complete inhibition of cdk2. Additional interactions between cdk2 and p21 also contribute to the inhibition of cdk2 [4,6], but the exact composition of the p21–cdk2 complexes has yet to be defined.

is specific for p27 and did not cross-react with $p21^{cip1}$ in muscle cell lysates (see Figs. 4–6). Our finding of p27 involvement in muscle development is consistent with the high level of p27 expression in skeletal muscle tissues [24] and with studies demonstrating that mice deficient in p27 expression grew to a bigger size than normal [34–36]. This conclusion is further validated by a recent report, which appeared after the completion of our study and which demonstrated that p27 is upregulated transiently during myogenic differentiation in vivo [37]. Since the increase in p27 expression occurred rapidly prior to the overt expression of differentiated functions, we believe that its increase may contribute to the initial withdrawal from the cell cycle that allows muscle differentiation to proceed. The other cdk inhibitors may then function in the differentiated cells to maintain the permanent postmitotic state.

Among the most interesting questions raised by the current study are the unexpected and apparently coordinated upregulation of cyclin D3 and $p27^{kip1}$, and their increased association with cdk2 (illustrated in Fig. 7). What role may this reorganization of cdk2 complexes play in the myogenic differentiation process? One possibility is that the simultaneous increase in cyclin D3 and p27 expression may serve to sequester cdk2 from cyclin A by favoring the formation of D3–cdk2–p27 complexes (see Fig. 7), since cyclin D3 interacts more strongly with p27 relative to cyclin A [24], and the ternary cyclin–cdk2–p27 complexes are more stable than the binary cyclin–cdk2 complexes [38,39]. Such a reorganization of cyclin–cdk2 complexes may be responsible for the elimination of the cyclin A-mediated cdk2 activity. Another intriguing possibility is that the binding to D3 and p27 may redirect cdk2 to other potential target substrates. In this regard, the existence of active cyclin–cdk complexes that contain associated cdk inhibitors is well documented [39,40] and a recent report has demonstrated the p27 protein could indeed modulate the substrate specificity of its associated cyclin–cdk complexes [41]. It is also interesting to note that cyclin D–cdk2 complexes exhibit in vitro substrate specificity that is distinct from either cyclin E–cdk2 or cyclin D–cdk4 [42]. Thus, it is not too surprising that the cyclin D3–cdk2–p27 complex in differentiated muscle cells (see Fig. 7) might retain residual activity towards specific substrates with functions unrelated to cell cycle progression. In partial support of this idea is the finding that overexpression of cyclin D1,

but not cyclin D3, efficiently inhibits myogenic differentiation and muscle-specific gene expression [43,44].

In summary, while the precise roles of cyclin D3 and p27 in the muscle differentiation process still need to be elucidated, it is becoming clear that the inhibition of cell proliferation accompanying terminal myogenic differentiation is orchestrated by the coordinated actions of multiple cdk inhibitors and that the concomitant reorganization of the existing cyclin–cdk complexes may also be critically important.

Acknowledgements

This work was supported by a Public Health Service Grant AR40682 from the National Institute of Arthritis, Musculoskeletal and Skin Diseases and grants from the Muscular Dystrophy Association and the University of Missouri-Columbia and Ellis-Fischel Cancer Center. We would like to thank Dr. Andrew Lassar for providing the MyoD expression plasmid pEMc11, and Ms. Danielle Lewis for her technical assistance in some of the studies.

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